

Yeast *MAK3* N-Acetyltransferase Recognizes the N-Terminal Four Amino Acids of the Major Coat Protein (*gag*) of the L-A Double-Stranded RNA Virus

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The *MAK3* gene of *Saccharomyces cerevisiae* encodes an N-acetyltransferase whose acetylation of the N terminus of the L-A double-stranded RNA virus major coat protein (*gag*) is necessary for viral assembly. We show that the first 4 amino acids of the L-A *gag* protein sequence, MLRF, are a portable signal for N-terminal acetylation by *MAK3*. Amino acids 2, 3, and 4 are each important for acetylation by the *MAK3* enzyme. In yeast cells, only three mitochondrial proteins are known to have the *MAK3* acetylation signal, suggesting an explanation for the slow growth of *mak3* mutants on nonfermentable carbon sources.

Most proteins made in eukaryotes are N-terminally acetylated, but the role of these modifications and the signals determining them are, in most cases, unclear (reviewed in references 5 and 17). There are at least three enzymes in *Saccharomyces cerevisiae* that perform this process. The enzyme responsible for most acetylations is encoded by two genes, *NAT1* (also called *AAA1*) and *ARD1* (7, 13). While *NAT1* encodes peptides of the purified major N-acetyltransferase (7), the sequence of *ARD1* (24) strongly suggests that it encodes a catalytic subunit of an acetyltransferase (21), and the requirement for both *NAT1* and *ARD1* in stoichiometric amounts suggests the enzyme has two subunits (13). Although the *NAT1-ARD1* system is responsible for acetylation of about 20% of all yeast proteins (10), mutants in either gene (or both) are viable, showing slowed growth, defects in repression of the HML mating locus, and failure to sporulate or to enter G₀ (9, 13, 24). This indicates that most proteins modified by this system do not need the modification for function. Based on in vitro and in vivo studies, the *NAT1 (AAA1)-ARD1* system modifies mainly proteins whose initiator methionine has been removed leaving an N-terminal serine, threonine, glycine, or alanine (8, 18, 20). Although changing a protein's second amino acid residue can change its acetylation either in vivo or in vitro (4, 8, 12, 23), this is clearly not the only determinant of acetylation (8, 17, 23).

A second yeast N-acetyltransferase, capable of modifying N-terminal methionine, has also been described (11). This activity is present in mutants lacking the *NAT1-ARD1* activity, and, based on data to date, seems to prefer a penultimate D, E, or N residue (11, 18).

The *MAK3* gene encodes a protein methionine N-acetyltransferase responsible for the acetylation of the N-terminus of *gag*, the major coat protein of the L-A double-stranded RNA virus of *S. cerevisiae* (21, 22). This modification is necessary for assembly of L-A virus particles (22). Mutants with point or deletion mutations in *mak3*, independent of their inability to propagate the L-A virus, show slow growth on nonfermentable carbon sources, suggesting that the

MAK3 N-acetyltransferase is also responsible for modifying one or more proteins involved in respiration (1, 21).

We previously showed that the first 13 amino acids of the L-A major coat protein, when attached to the N-terminus of β -galactosidase, are a sufficient signal to allow N-acetylation of the fusion protein by *MAK3* and to block N-acetylation of the β -galactosidase by other yeast systems (22). In this work, we further dissected the specificity of the *MAK3* activity, and, on the basis of the results, suggest an explanation for the respiration defect of *mak3* mutants.

pJC11B is the CEN vector pRS316 containing the *URA3* marker gene, into which a 5.4-kb segment of yeast chromosomal DNA containing the *MAK3* gene and promoter has been inserted (21).

To study the requirements of *MAK3* N-terminal acetylation, we used pJC28, carrying a 2 μ m DNA replicon, the yeast *TRP1* gene as a selectable marker, and the *PGK1* promoter driving production of a 110-kDa fusion protein consisting of the 13 N-terminal residues of the L-A major coat protein, nine connecting amino acids encoded by a multiple cloning site, and β -galactosidase (22). The structure of pJC28 around the translation start site is as follows:

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EcoRI
GAATTGCATAGAAA ATG CTA AGA TTT GTT ACT AAA AAC TCT CAA GAT AAA TGG
          M   L   R   F   V   T   K   N   S   Q   D   K   S
          |<--- N-Terminal 13 amino acids from L-A gag ----->|

          XbaI BglII   XhoI       SmaI KpnI
AAT TCT AGA TCT CGA GCC CGG GGT ACC GAT CCC GTCGTTTACAAACGT...
N   S   R   S   R   A   R   G   T   D   P   V...β-galactosidase
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JD81 and JD99 were made by modification of pF'8 (2), substituting the region between the initiator methionine and the slippery site (the site of the -1 ribosomal frameshift) with sequence coding for LRFG and LRFVT, respectively. Site-directed mutagenesis of pJC28 and pF'8 was done as described by Kunkel (6) with the Muta-Gene kit from Bio-Rad and oligonucleotides synthesized on an Applied Biosystems 381A oligonucleotide synthesizer. All mutations were confirmed by DNA sequence analysis.

A *mak3-1* strain (Y48 = *MATa trp1-Δ ura3-52 aro7 mak3-1*) and the *mak3-1* strain with the *MAK3* plasmid, pJC11B (21) (*MAK*⁺ host) were transformed with pJC28 and its derivatives. The fusion proteins were purified on Proton columns (Promega), containing a monoclonal anti-

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TABLE 1. N-acetylation of *gag-lacZ* fusion proteins in *MAK3* and *mak3* hosts^a

Construct	N terminus of fusion protein														<i>mak3</i> host	<i>MAK</i> ⁺ host
	1	2	3	4	5	6	7	8	9	10	11	12	13	14		
JC28	M	L	R	F	V	T	K	N	S	Q	D	K	S	n....	Unblocked	Blocked
JC29	M	L	R	F	V	T	K	N	S	Q	n	s	r	s....		Blocked
JC30	M	L	R	F	V	T	K	n	s	r	s	r	a	r....		Blocked
JC31	M	L	R	F	n	s	r	s	r	a	r	g	t	d....	Unblocked ^b	Blocked
JC32A	M	L	A	F	V	T	K	N	S	Q	D	K	S	n....	Unblocked	Blocked
JC32B	M	L	E	F	V	T	K	N	S	Q	D	K	S	n....		Unblocked
JC33A	M	L	R	A	V	T	K	N	S	Q	D	K	S	n....	Unblocked	Blocked
JC33B	M	L	R	E	V	T	K	N	S	Q	D	K	S	n....		Unblocked
JC34A	M	L	R	F	A	T	K	N	S	Q	D	K	S	n....	Blocked	Blocked
JC34B	M	L	R	F	E	T	K	N	S	Q	D	K	S	n....	Unblocked	Blocked
JC35	M	S	R	F	V	T	K	N	S	Q	D	K	S	n....	Blocked	Blocked
JC36	M	W	R	F	V	T	K	N	S	Q	D	K	S	n....	Unblocked	Blocked
JC38	M	E	R	F	V	T	K	N	S	Q	D	K	S	n....	Blocked	Blocked
JC37	M	A	R	F	V	T	K	N	S	Q	D	K	S	n....	Unblocked ^c	Unblocked ^c

^a *gag-lacZ* fusion proteins with the sequences shown were synthesized in *mak3* or wild-type hosts. The fusion proteins were isolated and sequenced. Upper-case letters are from the N terminus of the L-A *gag* protein except for the underlined mutant residues. Lower-case residues are from the polylinker region and β -galactosidase. The methionine-cleaving system should remove the N-terminal methionine if the second amino acid is smaller than aspartate (e.g., S or A but not L, W, or E). The determined sequence confirmed the sequence predicted from the DNA sequence for each unblocked protein.

^b This total sample contained 12.5 μ g of protein (109 pmol of a 115-kDa protein), as determined by amino acid analysis, and 74.6 pmol of leucine was detected in the second cycle above a background of 2.8 pmol in cycle 1. This implies that 68% of the protein was sequencing, a high value for unblocked proteins blotted on PVDF membranes. This implies that nearly all of the molecules were unblocked.

^c Methionine removed.

body against β -galactosidase immobilized on Sepharose. Cells were grown in 40 ml of synthetic complete medium lacking tryptophan (16) overnight at 30°C to an optical density at 550 nm of 3 to 4, harvested by centrifugation, washed with lysis buffer (0.1 M Tris-HCl [pH 7.5], 10 mM EDTA), resuspended in 0.5 ml of the same buffer containing 2 mM phenylmethylsulfonyl fluoride, and lysed with glass beads. The lysate was centrifuged at 10,000 $\times g$, and the clear supernatant was precipitated with 3 volumes of saturated $(\text{NH}_4)_2\text{SO}_4$ for 1 h at 0°C. After centrifugation, the pellet was redissolved in 300 μ l of lysis buffer containing 2 mM phenylmethylsulfonyl fluoride, vortexed, and diluted five times with binding buffer (50 mM Tris-HCl [pH 7.5]). To this solution, 1 ml of wet Protosorb equilibrated with binding buffer was added. After incubation at 4°C for 1 h, the suspension was poured into a Bio-Rad disposable column and washed with 10 ml of binding buffer containing 0.2% Nonidet P-40 and 5 ml of binding buffer. The fusion protein was eluted from the column with 0.1 M NaHCO_3 - Na_2CO_3 (pH 10.8). A 1.5-ml fraction containing the fusion protein (optical density at 280 nm equals 0.15) was concentrated in a Centricon 30 (Amicon) concentrator and applied to a sodium dodecyl sulfate-7.5% polyacrylamide gel.

The gel was electroblotted to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) for 1 h at 300 mA in 10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (pH 11)-10% methanol. The bands were visualized with Coomassie brilliant blue and cut, and the protein (approximately 10 to 50 μ g) was sequenced directly on the PVDF membrane with a Sequemat Mini 15 sequencer by the W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University.

We previously reported that the first 13 amino acids of the L-A-encoded *gag* protein were sufficient, when placed at the N terminus of β -galactosidase, to direct acetylation by *MAK3* and prevent either removal of the initiating methionine or acetylation by the *NAT1-ARD1* system or any other N-modification system (22). The leucine in position 2 is predicted to prevent removal of the N-terminal methionine (19), and in our studies, the N-terminal methionine of *gag* was not removed (22). To further study the specificity of the

MAK3 enzyme, we made deletions or single amino acid changes in pJC28 in the N-terminal 13 residues that are derived from the N terminus of L-A's *gag*. The modified proteins were synthesized in either a *MAK3* or an isogenic *mak3* strain, purified, and sequenced. We measured acetylation by the *MAK3* enzyme as *MAK3*-dependent blockage to protein sequencing.

Inclusion of only 10, 7, or 4 amino acids of the *gag* N terminus (JC29, JC30, and JC31) resulted in blocked protein when synthesized in the wild-type host (Table 1), and the protein with 4 residues of *gag* was unblocked when synthesized in a *mak3* mutant strain. Quantitation of the results (Table 1, footnote b) indicates that most of the protein was unblocked, but we could not exclude the possibility that a small proportion of the protein was blocked. Changing residue 3 or 4 to alanine did not prevent acetylation by the *MAK3* enzyme (JC32A, JC33A), but changing either residue to glutamate prevented modification (JC32B, JC33B). In contrast, changing residue 5 to glutamate (JC34B, Table 1) left the protein a substrate only for the *MAK3* enzyme, consistent with the finding that the first four residues are sufficient to direct modification by *MAK3* (JC31, Table 1). Changing residue 5 to alanine made the protein capable of modification by another system (JC34A, Table 1).

Changing the second residue to alanine (JC37) resulted in loss of the terminal methionine, as predicted (19), and failure of modification by any acetyltransferase. Changing residue 2 to serine or glutamate (JC35, JC38) made the protein a substrate for a non-*MAK3* enzyme, and changing it to tryptophan (JC36) had no effect. Since serine or alanine as the second residue should result in removal of the initiator methionine, the acetylation of JC35 may be due to the *NAT1-ARD1* system. Acetylation of JC38 may be due to the methionine *N*^{acetyl}transferase, which was shown to act on peptides with acidic second residues (11, 18). Thus, residue 2 determines whether the *MAK3* enzyme will modify a protein, but this may be largely a reflection of methionine removal and the action of other acetyltransferases.

The results presented here suggest that residues 2, 3, and 4 are important in determining whether a protein will be

acetylated by the *MAK3* enzyme, although not all need be what they are in L-A's *gag*. We attempted to use this information to make an unblocked fusion protein using the site in the L-A dsRNA virus that promotes -1 ribosomal frameshifting (2, 3). Our goal was to confirm the protein sequence across the frameshift site. The expected sequences and the reading frames are shown below:

shift
JD99: 0 frame M L R F V T G L
R S G . . β -galactosidase -1 frame

JD81: 0 frame M L R F Q G L
R S G R . . β -galactosidase -1 frame

Both fusion proteins, synthesized in the same *mak3* mutant strain used for the studies described in Table 1 and affinity purified, were largely (though not completely) blocked, preventing our confirming their sequences. This, as well as the construct JC34A with a V→A change at position 5, indicates that the rules for acetylation in vivo by the yeast non-*MAK3* systems must involve more than recognition of just the first four amino acid residues which in each case are identical to L-A's *gag*.

When each of the proteins modified by the *MAK3* enzyme was purified from a *mak3* mutant, the unblocked sequence showed that the methionine had not been removed. The methionine *N*-acetyltransferase described by Lee et al. (11) appeared to prefer substrates whose second residue was acidic. The *MAK3*-independent acetylation of JC38 (MERFVT...) might be due to the enzyme studied by Lee et al. (11), but at least our result suggests there are two methionine *N*-acetyltransferases.

Since *MAK3* seems to recognize the N-terminal sequence MLRF..., we searched the protein data bases for such an N terminus. We found only three yeast proteins that begin with MLRF (and none beginning with MLAF, MLRA, or MWRF), α -ketoglutarate dehydrogenase (*KGD1*) (15), fumarate hydratase (*FUM1*) (25), and a 37-kDa mitochondrial ribosomal protein (*MRP1*) (14). These are all mitochondrial proteins, and this may explain the mild growth defect of *mak3* mutants on nonfermentable carbon sources (1, 21). The signal for *MAK3* acetylation would be part of the import signal sequence which is usually removed on entry into the mitochondrion. Presumably, acetylation is needed for optimal import of one of these proteins into the mitochondrion, not for its function once it arrives.

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